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IDENTIFICATION OF A SELENOCYSTEINE-SPECIFIC AMINOACYL TRANSFER RNA FROM RAT LIVER

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The aminoacylation of rat liver tRNA with selenocysteine was studied in tissue slices and in a cell-free system with [^{75}Se]selenocysteine and [^{75}Se]selenite as substrates. [^{75}Se]Selenocysteyl tRNA was isolated via phenol extraction, 1 M NaCl extraction and chromatography on DEAE-cellulose. [^{75}Se]Selenocysteyl tRNA was purified on columns of DEAE-Sephacel, benzoylated DEAE-cellulose and Sepharose 4B. In a dual-label aminoacylation with [^{35}S]cysteine, the most highly purified ^{75}Se-fractions were > 100-fold purified relative to ^{35}S. These fractions contained < 0.7% of the [^{35}S]cysteine originally present in the total tRNA. When [^{75}Se]selenocysteyl tRNA was purified from a mixture of ^{14}C-labeled amino acids, over 97% of the [^{14}C]aminoacyl tRNA was removed. The [^{75}Se]selenocysteine was associated with the tRNA via an aminoacyl linkage. Criteria used for identification included alkaline hydrolysis and recovery of [^{75}Se]selenocysteine, reaction with hydroxylamine and recovery of [^{75}Se]selenocysteyl hydroxamic acid and release of ^{75}Se by ribonuclease. The specificity of [^{75}Se]selenocysteine aminoacylation was demonstrated by resistance to competition by a 125-fold molar excess of either unlabeled cysteine or a mixture of the other 19 amino acids in the cell-free selenocysteine aminoacylation system.

Introduction

Several selenoproteins recently were shown to contain selenocysteine as the active selenium moiety [1–3]. In rat liver glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9), the selenocysteine is located within the peptide backbone [4]. Earlier investigations on cysteyl-selenocysteyl tRNA in *Escherichia coli* [5] concluded that selenocysteine charges species of cysteine-accepting tRNA in bacteria.

Work in this laboratory [6] showed that the incorporation of selenium into glutathione peroxidase is completely inhibited by either cycloheximide or puromycin and that a large amount of free selenocysteine is synthesized at the same time. One possible explanation for these findings is that selenocysteine is incorporated into glutathione peroxidase via an aminoacyl tRNA and protein synthesis. The purpose of this investigation was to determine if rat liver tRNA can be aminoacylated with selenocysteine, and if it can, whether the selenocysteine is charged to a specific selenocysteine-accepting tRNA.

Materials and Methods

Materials. Rats were male albinos of the Sprague-Dawley line (80–120 g and 6–8-weeks-

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old) from Simonsen Laboratories. Ribonuclease B (2000 units/mg) was obtained from Worthington Biochemicals; D, L-selenocystine and DEAE-Sephacel from Sigma Chemical Co.; L-[^{75}Se]-selenocystine (100–300 mCi/mmol) from Amersham Radiochemical Centre; and L-[^{35}S]cystine (> 300 Ci/mmol), [^{75}Se]selenite (9–35 Ci/mmol) and [^{3}H]iodoacetic acid (291 mCi/mmol) from New England Nuclear. The ^{14}C-labeled amino acids (equal specific activity mixture without cysteine, methionine, tryptophan, glutamine and asparagine) were purchased from ICN. Sephadex G-50, DEAE-Sephacel and Sepharose 4B were obtained from Pharmacia Fine Chemicals, and DEAE-cellulose (DE-32) from Whatman. Benzoylated DEAE-cellulose (Cellex B-D), Dowex AG-50WX8 and Dowex AG-1X8 were purchased from BioRad Laboratories.

Liver slice aminoacylation procedures. Rats were decapitated and bled, then the livers were excised and placed into ice-cold Hank's balanced salt solution. Slices of 0.8 mm thickness were prepared on a McIllwain tissue chopper, washed in ice-cold Hank's balanced salt solution, drained on filter paper and weighed. [^{75}Se]Selenocystine and [^{35}S]cystine were reduced in 100 mM dithioerythritol (pH 8.5) for 15 min at 50°C immediately before they were added to Hank's balanced salt solution. The mixture was then titrated to pH 7.8 with 7.5% $NaHCO_3$. Prior to incubation with shaking at 37°C for 30 min, 4 ml oxygen-saturated Hank's balanced salt solution were added to each g of liver slices. The final concentrations were 40 μM [^{75}Se]selenocysteine, 164 nM [^{35}S]cysteine, 20 μM each of 19 unlabeled amino acids (without cysteine) and 2 mM dithioerythritol. Batch size varied from 5–50 g of slices.

An alternative procedure for the preparation of singly-labeled [^{75}Se]selenocysteyl tRNA at high specific activity was also used with liver slices. This procedure was identical to the above except that the only labeled compound added was [^{75}Se]selenite at a concentration of 1 μM and dithioerythritol was omitted. In this procedure, a mixture of all 20 amino acids was added to the incubation mixture at a concentration of 20 μM each.

Isolation of aminoacyl tRNA. Isolation of tRNA was done by a modification of the procedure described by Sein et al. [7]. After incubation, liver slices were washed once in 0.1 M sodium acetate (pH 4.5)/1 M NaCl/5 mM EDTA (Buffer A). They were then homogenized in a blendor for 90 s with 1.5 vol. Buffer A (v/w) and 1.5 vol. 80% water-saturated phenol that contained 0.1% 8-quinolinol (w/w). The phases were separated by centrifugation at 15 000 × *g* for 15 min. Each phase was reextracted once with 1.5 vol. of the other phase. The first phenolic phase was discarded and the first aqueous phase was saved. The second phenolic and aqueous extracts were mixed and extracted against each other. The two aqueous extracts were pooled, and the nucleic acids were precipitated by adding 3 vol. 95% ethanol and allowing the mixture to stand for at least 3 h at −15°C. After centrifugation at 8000 × *g* for 5 min, the precipitated pellet was suspended in 0.8 ml Buffer A/g of slices and recentrifuged at 8000 × *g* for 5 min. The supernatant was saved and the pellet was resuspended in 0.4 ml Buffer A/g of slices and centrifuged again. The supernatant fractions were combined and diluted with three times their volume of 13 mM $MgCl_2$/1.3 mM Na_2EDTA. This extract was loaded onto a column of DEAE-cellulose (1.5-cm diameter with a 0.5-ml bed volume/g of slices) equilibrated with 0.25 M NaCl/10 mM $MgCl_2$/1 mM Na_2EDTA (Buffer B). The column was washed with Buffer B until the absorbance of the eluate at 260 nm was equal to the background absorbance. The tRNA was eluted with 0.7 M NaCl/10 mM $MgCl_2$/1 mM Na_2EDTA (Buffer C), and those fractions with significant ultraviolet absorbance were pooled.

Final intracellular specific radioactivities. An aliquot of labeled liver slices from an incubation with [^{35}S]cysteine and [^{74}Se]selenocysteine was homogenized in a glass-Teflon homogenizer with 1.5 vol. distilled water immediately after completion of the incubation. 3 vol. acetone were added and the mixture was stored overnight at −15°C. The bulk of the protein was removed by centrifugation at 15000 × *g* for 20 min, and the supernatant was evaporated to dryness at 37°C under vacuum. The residue was dissolved in a small aliquot of water and then mixed with an equal volume of 6% 5′-sulfosalicylic acid. The resultant precipitate was removed by centrifugation, and the supernatant was adjusted to pH 2–2.2 with 1 M LiOH. This

solution was loaded onto a Beckman 120B amino acid analyzer column in order to quantitate cysteine. The amount of ^{35}S in cysteine was measured by continuous-flow scintillation detection of the amino acid analyzer column eluant. The ^{35}S peak at the elution time of cysteine was integrated and divided by the amount of cysteine (nmol) as determined by reaction with ninhydrin. The specific radioactivity of [^{75}Se]selenocysteine was assumed to be insignificantly changed by dilution with endogenous selenocysteine and was only corrected for decay.

Sepharose-4B chromatography. Sepharose-4B chromatography was performed according to the method of Hatfield [8]. Singly- or doubly-labeled [^{75}Se]selenocysteyl tRNA from a liver slice incubation was prepared as described above. The tRNA fraction from DEAE-cellulose or B-D cellulose chromatography was made 1.5 M in $(NH_4)_2SO_4$ by addition of 4 M $(NH_4)_2SO_4$. The sample was then applied to a 1.5 × 30 cm column of Sepharose 4B in 1.5 M $(NH_4)_2SO_4$ prepared in 10 mM sodium acetate (pH 4.5)/10 mM $MgCl_2$/10 mM Na_2EDTA/6 mM mercaptoethanol (Buffer D). The column was washed with Buffer D until the absorbance of the eluate at 260 nm was negligible. The tRNA was eluted with a negative linear gradient from 1.5–0 M $(NH_4)_2SO_4$ in Buffer D in a total volume of 500 ml and at a flow rate of 0.6 ml/min. 3-ml fractions were collected, precipitated and counted for radioactivity as described below.

Analysis of chromatographic fractions. Fractions from chromatography of labeled tRNA were collected and mixed with 0.2 mg yeast sRNA, 50% trichloroacetic acid was added to a final concentration of 8%, and the tube contents were mixed and left on ice for at least 15 min before they were filtered through 0.45 μm Millipore filters. The precipitates were washed twice with cold 5% trichloroacetic acid and once with 70% ethanol, then they were dried in scintillation vials under an infrared lamp. A 10-ml aliquot of Aquasol (New England Nuclear) was added to each sample. The samples were counted for ^{75}Se on a Packard Tri-Carb NaI scintillation counter and for total radioactivity ($^{35}S + ^{75}Se$) on a Nuclear Chicago Mark II liquid scintillation counter. The net radioactivity of ^{35}S was determined by subtracting the contribution from ^{75}Se as determined by proportion from the γ-counting data.

Synthesis of [^{3}H]carboxymethylselenocysteine ethyl ester. A 20-mg sample of D, L-selenocysteine was mixed with 4 ml absolute ethanol and 75 μl concentrated H_2SO_4 and refluxed for 12 h. After addition of 2 ml distilled water, concentrated KOH was added to adjust the mixture to pH 8.0. Then, 40 mg $NaBH_4$ were added and dissolved. After 5 min, 1 mCi [^{3}H]iodoacetic acid (0.6 mg) and 20 mg sodium iodoacetate were added and the mixture was allowed to stand for 20 min at room temperature. Concentrated HCl was added to pH 1, and the mixture was adsorbed onto a 5-ml column of Dowex AG-50WX8 (H^+ form in 0.1 M HCl). The Dowex AG-50 column was washed with 10 ml 0.1 M HCl and 20 ml distilled water. The carboxymethylselenocysteine ethyl ester was eluted with 20 ml 2 M NH_4OH directly onto a 5-ml column of Dowex AG-1X8 (OH^- form in water). The Dowex AG-1X8 column was washed with 20 ml water and eluted with 20 ml 2 M acetic acid. The final eluant was evaporated to dryness under vacuum and dissolved in 0.18 ml distilled water. This solution was used as the [^{3}H]carboxymethylselenocysteine ethyl ester standard. The yield of the ester, based on the recovery of ^3H after amino acid analysis, was estimated at 54%.

Cell-free aminoacylation. Rats were fasted for 18 h before they were decapitated. The livers were removed and placed in ice-cold 0.25 M sucrose/10 mM Tris-HCl (pH 7.6)/0.1 mM EDTA. The livers were weighed, minced with scissors and then homogenized in a glass-Teflon homogenizer with 2 vol. of the same buffer. The homogenate was centrifuged for 90 min at 66000 × *g*, and the entire supernatant portion was decanted and saved. The cytosol was then dialyzed for 6 and 18 h against 2 changes of 30 vol. 50 mM Tris-HCl (pH 7.5 at 37°C)/50 mM KCl/20 mM $MgCl_2$/10 mM mercaptoethanol at 0–4°C.

[^{75}Se]Selenocysteine was prepared by first drying [^{75}Se]selenocystine (in 1 M HCl) under nitrogen at 45°C and then adding 60 μl mercaptoethanol/125 μl 2 M NH_4OH/1.025 ml $NaBH_4$ (1 mg/ml) per mg of [^{75}Se]selenocystine. The mixture was heated 5 min at 45°C and then dried under nitrogen at 45°C. The [^{75}Se]selenocysteine was then dissolved in 0.01 M HCl/1 mM

dithioerythritol at a concentration of 5 mM and used within 1 h.

Unlabeled cysteine was prepared by heating a solution of 25 mM cystine and 100 mM dithioerythritol, at pH 9 and 45°C, for 15 min. This solution was diluted appropriately with 100 mM dithioerythritol (pH 9) to make stock solutions of 0, 0.2, 0.5, 1, 2, 5, 10 and 50 mM cysteine in 100 mM dithioerythritol. Stock solutions of a mixture of 19 unlabeled amino acids (without cysteine) were prepared at concentrations of 0, 0.2, 0.5, 1, 2, 5, 10 and 50 mM each in 1 M HCl. When used, the 19 amino acid stock solutions were added to the tubes first with an appropriate amount of 1 M NaOH to bring them to pH 7, and the rest of the components were then added.

Each 2.5–4.5-ml incubation mixture contained per ml of cytosol dialyzate: 0.1 ml 50 mM Na_2ATP (pH 7.0)/125 μl 19 amino acid-stock solution (0–50 mM) or 24 μl of 2.5 mM each of 19 unlabeled amino acids (no cysteine) plus 125 μl cysteine stock solution. The reaction was initiated by addition of 8 μl [^{75}Se]selenocysteine per ml incubation mixture (40 μM final concentration), and incubation was carried out in stoppered tubes with shaking at 37°C for 30 min. After incubation, the tubes were placed in ice and 0.1 ml 2.5 M sodium acetate (pH 4.5) was added per ml dialyzate. Then, 1.3 ml 80% water-saturated phenol that contained 0.1% 8-quinolinol (w/w) was added per ml of dialyzate. After vigorous shaking for 1 min, the extracts were centrifuged for 20 min at $22000 \times g$, and the aqueous layer was removed and saved. The phenol was reextracted with an equal volume of Buffer A, recentrifuged and the aqueous layers were combined. The samples were precipitated with 3 vol. 95% ethanol for 3 h at -15°C and centrifuged for 10 min at $15000 \times g$. The precipitates were redissolved in 0.6 ml Buffer B per ml dialyzate and applied to miniature (5-ml bed vol.) columns of DEAE-cellulose equilibrated with Buffer B. The columns were washed with 30 ml of the same buffer. [^{75}Se]Selenocysteyl tRNA was eluted in 10 ml of Buffer C. The total ^{75}Se was divided by the total number of A_{260} units to give cpm of $^{75}Se/A_{260}$ of tRNA. 1 A_{260} unit is the amount of tRNA in 1 ml water that gives absorbance of 1 at 260 nm in a 1-cm cuvette. This ratio (cpm of $^{75}Se/A_{260}$) allowed comparisons of the relative charging levels in the different samples without regard to the recoveries of tRNA. For comparisons between experiments, these values were expressed as a percentage of the controls without added unlabeled amino acids.

Results

Identification of aminoacyl linkage

[^{75}Se]Selenocysteyl tRNA from a liver slice incubation with [^{75}Se]selenocysteine was partially purified on B-D cellulose and used without further purification for the following characterizations. [^{75}Se]Selenocysteyl tRNA was treated for 20 min in 12 μg/ml RNAase B and chromatographed on Sephadex G-50. Before ribonuclease treatement, 66% of the ^{75}Se eluted with the tRNA near the column void volume. After ribonuclease treatment, all of the ^{75}Se was found at the column total volume, showing that the ^{75}Se eluting near the void volume had indeed been bound to RNA.

Incubation of [^{75}Se]selenocysteyl tRNA for 30 min at 37°C and pH 8.5 released 94% of the ^{75}Se, whereas a control incubated for 30 min at 37°C and pH 4.5 released only 3% of the ^{75}Se. This lability towards mild alkaline hydrolysis is characteristic of aminoacyl tRNAs. The deacylated ^{75}Se was mixed with selenocystine, treated with iodoacetic acid plus $NaBH_4$ and then assayed for carboxymethyl [^{75}Se]selenocysteine by amino acid analysis. When corrected for losses in the recovery of carrier selenocystine, 24% of the ^{75}Se from [^{75}Se]selenocysteyl tRNA was accounted for by carboxymethyl[^{75}Se]selenocysteine. Although these recoveries seem low, they are typical for selenium compounds due to the instability of selenols at alkaline pH and the difficulty of obtaining complete reduction and carboxymethylation.

The reaction of [^{3}H]carboxymethylselenocysteine ethyl ester with 2 M NH_2OH resulted in the appearance of a single peak of ^{3}H at an elution time of 141 min on the amino acid analyzer. This peak was presumably the hydroxamic acid of carboxymethylselenocysteine. NH_2OH treatment caused the release of 91% of the ^{75}Se in tRNA, and 39% of the ^{75}Se released from [^{75}Se]selenocysteyl tRNA by NH_2OH treatment eluted with the ^{3}H marker at 141 min on the amino acid analyzer

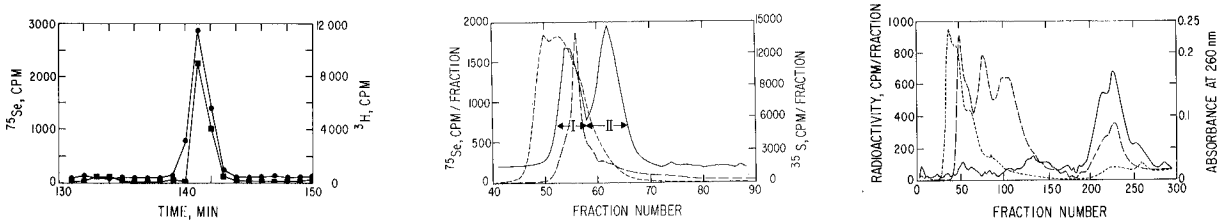

Fig. 1. Portion of the amino acid analyzer chromatogram of the reaction products of hydroxylamine plus carboxymethyl[^{75}Se]selenocysteyl tRNA and [^{3}H]carboxymethylselenocysteine ethyl ester. The ^{75}Se-labeled tRNA and ^{3}H-labeled ester were mixed first, then reacted with freshly redistilled hydroxylamine. The ^{3}H label shows the position of the resulting carboxymethylselenocysteine hydroxamic acid internal marker. ^{75}Se (●) was counted on the γ-counter and ^{75}Se+^{3}H were counted by liquid scintillation. ^{3}H (■) was determined by subtracting the contribution from ^{75}Se as determined by proportion from the γ-counting data.

Fig. 2. Typical DEAE-Sephacel chromatography of ^{35}S- and ^{75}S-labeled tRNA. Crude, unfractionated tRNA was prepared from liver slices incubated with [^{35}S]cysteine and [^{75}Se]selenocysteine as described in Materials and Methods. 127 A_{260} units of tRNA that contained 34000 cpm of ^{75}Se and 65500 cpm of ^{35}S were applied to a 1.5×90-cm column of DEAE-Sephacel in 0.38 M NaCl/10 mM $MgCl_2$/1 mM Na_2EDTA at 4°C; the flow rate was 0.5 ml/min. Elution was performed with a linear gradient of 0.38–0.73 M NaCl in 10 mM $MgCl_2$/1 mM Na_2EDTA in a total vol. of 800 ml. The absorbance at 260 nm (------) was measured by continuous ultraviolet monitoring and trichloroacetic acid-precipitable ^{35}S (— — —) and ^{75}Se (———) were measured as described in Materials and Methods.

Fig. 3. Example of a B-D cellulose chromatography of ^{35}S- and ^{75}Se-labeled tRNA conducted by a modification of the procedure of Nishimura and Weinstein [9]. Doubly-labeled tRNA was prepared from liver slices incubated with [^{35}S]cysteine and [^{75}Se]selenocysteine as described in Materials and Methods. 82 A_{260} units of tRNA that contained 50000 cpm of ^{75}Se and 61000 cpm of ^{35}S were applied to a 1.5×30-cm column of B-D cellulose in 0.4 M NaCl, 50 mM sodium acetate (pH 4.5) and 8 mM magnesium acetate at 4°C. Elution at a flow rate of 1 ml/min was started with a linear gradient of 0.4 to 1.1 M NaCl in 1.8 l (fraction No. 1 to No. 186) and was completed with a colinear gradient of 1.1 M NaCl and 0% ethanol to 1.8 M NaCl and 15% ethanol in 1 l. The absorbance at 260 nm (------) was measured in each fraction, and trichloroacetic acid-precipitable ^{35}S (— — —) and ^{75}Se (———) were measured as described in Materials and Methods. The ^{35}S and ^{75}Se profiles were smoothed by computer using an average of three adjacent points on either side, weighted by the coefficients of a binomial expansion with seven terms.

(Fig. 1). This relatively high yield of hydroxamic acid from [^{75}Se]selenocysteyl tRNA, 39%, compared to that from the ethyl ester, 2.9%, is also characteristic of aminoacyl tRNAs.

Dual-label aminoacylation with [^{35}S]cysteine

In order to measure the amount of cysteine attached to selenocysteyl tRNA, an experiment using 60 μCi [^{35}S]cysteine/ml and 10 μCi [^{75}Se]selenocysteine/ml was performed. In this experiment, the crude ^{35}S plus ^{75}Se-labeled tRNA from 5 g liver slices was first analyzed by chromatography on DEAE-Sephacel under the conditions described in Fig. 2. On this column, the [^{75}Se]selenocysteyl tRNA eluted in two well-separated peaks, each of which was separately pooled and diluted 1 : 1 with 0.1 M sodium acetate (pH 4.5) and 6 mM magnesium acetate before it was loaded onto a B-D cellulose column. B-D cellulose chromatography was conducted for both samples under the conditions described in Fig. 3. Each pooled fraction from the DEAE-cellulose chromatography eluted as a single peak from the B-D cellulose column. The ^{75}Se-containing fractions from these two chromatographies were again pooled separately and concentrated to approx. 3 ml on an Amicon UM-2 ultrafiltration membrane. 4 M $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M and the samples were analyzed by Sepharose 4B chromatography as described in Materials and Methods. The ^{75}Se from DEAE-Sephacel Peak I eluted from Sepharose 4B as a single peak at 0.5 M $(NH_4)_2SO_4$. The ^{75}Se from DEAE-Sephacel Peak II eluted from Sepharose 4B as two peaks. Peak IIA eluted at 1.36 M and Peak IIB eluted at 0.96 M $(NH_4)_2SO_4$. Peak IIB was subsequently pooled, reconcentrated and rechromatographed isocratically on Sepharose 4B in 0.77 M $(NH_4)_2SO_4$.

The final intracellular specific activities in this

TABLE I

PARTIAL PURIFICATION OF [^{75}Se]SELENOCYSTEYL tRNA FROM RAT LIVER SLICES INCUBATED WITH [^{75}Se]SELENOCYSTEINE AND [^{35}S]CYSTEINE

tRNA from 50 g of liver slices was purified using analytical DEAE-Sephacel, B-D cellulose and Sepharose 4B chromatographies as described in the legends of Figs. 2 and 3, Materials and Methods, and Results.

Purification step	Total tRNA (A_{260})	Total ^{75}Se		Total ^{35}S		Mol percent (Se/S + Se)	Enrichment of ^{75}Se (-fold purification of [^{75}Se]SeCys [a] tRNA relative to [^{35}S]Cys)
		(cpm)	(pmol)	(cpm)	(pmol)		
Crude tRNA [b]	99	11170	158	227350	4659	3.3	1
DEAE-Sephacel Peak I	42	1984	28	154350	3163	0.9	0.3
DEAE-Sephacel Peak II	12	3202	45	57620	1181	3.8	1.1
B-D Cellulose Peak I	14	239	3.3	0 [c]	—	—	—
B-D Cellulose Peak II	1.8	1985	28	7083	145	16.2	5.7
Sepharose 4B Peak IIA	0.4	762	10.8	92 [c]	1.9	85.8	168
Sepharose 4B Peak IIB	0.3	1127	15.9	172 [c]	3.5	82.0	134

[a] SeCys: selenocysteine.

[b] Fraction obtained between 0.25 and 0.7 M NaCl elution of first DEAE-cellulose column.

[c] ^{35}S-labeled tRNA was measured by liquid scintillation counting after precipitation with trichloroacetic acid and collection on Millipore filters as described for chromatographic fractions in Materials and Methods.

experiment were 48800 cpm/nmol for [^{35}S]cysteine and 70741 cpm/nmol for [^{75}Se]selenocysteine. The purification data for this experiment are shown in Table I. If the values for ^{35}S in Peaks IIA and IIB are summed and then corrected by the recovery of ^{75}Se: 1127 cpm + 762 cpm/11170 cpm = 0.169, then the total [^{35}S]cysteine accounted for in [^{75}Se]selenocysteyl tRNA is: ((92 + 172)/0.169)/227348, or 0.7% of the initial ^{35}S. In other words, no more than 0.7% of all the [^{35}S]-cysteine originally present in the crude tRNA was aminoacylated to selenocysteine-accepting tRNA. When the molar abundances of selenocysteine and cysteine in the purified ^{75}Se-tRNA fractions were calculated, Peaks IIA and IIB were found to contain 85.8 and 82.0% selenocysteine, respectively, relative to the total (S + Se) cysteine. These data show that only a small amount of cysteine could have been aminoacylated to these two forms of selenocysteine-accepting tRNA.

Dual-label aminoacylation with ^{14}C-labeled amino acids

In order to show that selenocysteine was not attached to a tRNA specific for another amino acid, [^{14}C]aminoacyl tRNA was prepared from a mixture of 15 ^{14}C-labeled amino acids in 26 ml of the cell-free aminoacylation mixture described in Materials and Methods, except that the ^{14}C-labeled amino acids were added at 1 μM each and no selenium compounds were added. The ^{14}C-labeled tRNA was mixed with [^{75}Se]selenocysteyl tRNA that was prepared in a liver slice incubation with 1 μM [^{75}Se]selenite and 20 μM each of 20 unlabeled amino aids. This strategy helped assure that the aminoacylation of [^{75}Se]selenocysteyl tRNA was not due to an excess of selenium. The ^{75}Se- and ^{14}C-labeled tRNA mixture was then purified on columns of DEAE-Sephacel, B-D cellulose and Sepharose 4B as described for the [^{35}S]cysteine dual-label tRNA purification except that 5 mM mercaptoethanol was added to all of the buffers. All of the [^{75}Se]selenocysteyl tRNA eluted as a single peak under these conditions, corresponding to Peak IIB in Table I. [^{75}Se]Selenocysteyl tRNA prepared from [^{75}Se]selenite released 91.9% of the ^{75}Se in 20 min at 37°C and pH 8.5, from which 16.9% was recovered as [^{75}Se]selenocysteine on the amino acid analyzer.

The purification data for this experiment are shown in Table II. The most highly purified ^{75}Se fraction was 62-fold purified relative to A_{260}, and over 97% of the ^{14}C-labeled tRNA had been removed. This result showed that the selenocys-

TABLE II

PARTIAL PURIFICATION OF [^{75}Se]SELENOCYSTEYL tRNA FROM A MIXTURE OF [^{14}C]AMINOACYL tRNAS

[^{75}Se]Selenocysteyl tRNA from a liver slice incubation with [^{75}Se]selenite was mixed with [^{14}C]aminoacyl tRNAs from a cell-free aminoacylation with an equal specific activity mixture of 15 [^{14}C]amino acids. The ^{75}Se in this mixture was purified using analytical DEAE-Sephacel, B-D cellulose and Sepharose 4B chromatographies as described in Materials and Methods and the legends of Figs. 2 and 3.

Purification step	Total tRNA (A_{260})	Total ^{75}Se (cpm)	Total ^{14}C (cpm)	^{14}C removed (%)	Purification (-fold relative to A_{260})
Crude tRNA	103	15080	168300	0	1
Post-DEAE-Sephacel	31	13090	39800	68.8	2.5
Post-B-D Cellulose	4.7	8080	5200	95.5	12
Post-Sepharose 4B	0.77	7010	2400	97.0	62

teine was not attached to a tRNA that normally accepts any of the 15 amino acids in the ^{14}C mixture. The specificity of aminoacylation was further indicated by the fact that the selenocysteine was attached to only one form of tRNA when it was charged in the presence of 1 μM [^{75}Se]selenite and 20 μM each of 20 amino aicds, whereas three forms of ^{75}Se-tRNA were observed at 40 μM selenocysteine and 20 μM of each of the other amino acids.

Competition by unlabeled cysteine

In order to test the ability of unlabeled cysteine to compete with [^{75}Se]selenocysteine for selenocysteine-accepting tRNA, the cell-free aminoacylation system described in Materials and Methods was developed. Three identical incubations were conducted at each of eight cysteine concentrations (0, 20, 50, 100, 200, 500, 1000 and 5000 μM). Table III shows the charging level (cpm ^{75}Se/A_{260}) as a percentage of the controls at different cysteine

TABLE III

COMPETITION OF SELENOCYSTEINE AMINOACYLATION BY CYSTEINE AND A MIXTURE OF 19 AMINO ACIDS

The expected values were calculated according to the formula: expected percentage = 40 μM ÷ (40 μM + added + endogenous) × 100%. Endogenous concentrations in rat liver of 100 μM cysteine and 5 mM average amino acids were assumed. [^{75}Se]Selenocysteine was incubated in the cell-free aminoacylation system described in Materials and Methods with increasing concentrations of the indicated unlabeled amino acids. Data are the means of three samples ± S.D. except as noted.

Concentration of added amino acids (μM)	Selenocysteine charging level (% of control ^{75}Se cpm/A_{260})			
	+ cysteine		+ 19 amino acids	
	expected	actual	expected	actual
0	100	100 ± 22	100	100 ± 20
20	66.7	95.5 ± 7.1	67.7	98.8 ± 7.9
50	44.4	70.9 ± 15	45.5	101 ± 5.5
100	28.6	70.8 ± 14 [a]	29.5	130 ± 8.5
200	16.6	70.0 ± 2.2 [a]	17.3	135 ± 14
500	7.43	75.5 ± 11	7.72	154 ± 15
1000	3.86	73.6 ± 2.3	4.02	147 ± 2.2
5000	0.796	48.7 ± 18	0.829	760 ± 159

[a] Mean of two determinations ± one-half the range.

concentrations. The theoretical data that would be expected if cysteine and selenocysteine competed equally for the same acceptor tRNAs, and assuming a 100 μM cysteine concentration in rat liver, is also shown in this table. The major features of this table are: (i) the observed competition at all the cysteine concentrations is much less than would be expected if cysteine and selenocysteine were competing equally for the same acceptor tRNAs, and (ii) there was no significant change in the [^{75}Se]selenocysteyl tRNA charging level from 50–1000 μM cysteine — a 20-fold variation in cysteine levels that includes the range of physiological concentrations in rat liver. It is likely that at sufficiently low cysteine concentrations and relatively high selenocysteine concentrations (40 μM), the selenocysteine can aminoacylate some of the cysteine-acceptor tRNA, and this might account for the 28% decrease between the ranges of 0–20 μM and 50–1000 μM cysteine. Since physiological cysteine concentrations are always 100–10000-times higher than physiological selenocysteine concentrations [10], only a tiny fraction of the total cysteine-accepting tRNA could ever be acylated with selenocysteine.

On the other hand, if one compares the [^{75}Se]selenocysteyl tRNA charging levels at 0 and 5000 μM cysteine, there is an apparent inhibition of [^{75}Se]selenocysteine aminoacylation of about 50%. Based upon an assumed concentration of cysteine in rat liver of 100 μM, the concentration of cysteine in the dialyzed rat liver cytosol was estimated at 100 μM/(30 × 30) after dialysis, which equals 0.11 μM. Therefore, the molar ratio of cysteine to selenocysteine is $\leq 1:360$ in the controls without added cysteine and $\geq 125:1$ in the 5000 μM cysteine samples. This represents at least a 45000-fold increase in the cysteine to selenocysteine molar ratio, yet this great increase caused only a 50% decrease in the [^{75}Se]selenocysteine aminoacylation level.

Competition by a mixture of 19 unlabeled amino acids

In order to show that the aminoacylation of selenocysteine was not due to mischarging of some tRNA other than those tested in the dual-label experiments, a competition experiment was conducted using the cell-free aminoacylation system described in Materials and Methods with increasing concentrations of a mixture of 19 unlabeled amino acids (without cysteine). Triplicate incubations were conducted at 0, 20, 50, 100, 200, 500, 1000 and 5000 μM each of the added 19 unlabeled amino acids. The results are shown in Table III with the data that would be expected assuming a 5-mM average amino acid concentration in rat liver and equal affinities for the aminoacyl tRNA synthetase. This mixture of 19 amino acids was completely ineffetive as a competitor of selenocysteine aminoacylation. From these data, it was estimated that the aminoacylation of selenocysteine has a selectivity of at least 1000 to 1 with respect to these amino acids. Since selenocysteine is acylated to this tRNA in preference to all 20 of the protein amino acids, then it must be considered a specific selenocysteine-accepting tRNA.

Discussion

The main supporting evidence for the conclusion that rat liver contains a form of tRNA that is aminoacylated specifically with selenocysteine is: (i) there are chromatographically distinct forms of [^{75}Se]selenocysteyl tRNA that can be easily separated from > 99% of the [^{35}S]cysteyl tRNA and from 97% of the [^{14}C]aminoacyl tRNA; (ii) the chemical form of selenocysteyl tRNA is an active aminoacyl linkage as shown by digestion by ribonuclease, deacylation at pH 8.5 and recovery of carboxymethylselenocysteine, and the formation of carboxymethylselenocysteine hydroxamic acid with NH_2OH; (iii) the selectivity of the selenocysteine aminoacylation reaction is at least 125 to 1 in favor of selenocysteine relative to cysteine and is even greater relative to the other 19 amino acids.

These results are in contrast to reports of similar experiments with *E. coli* [5] where only slight and reportedly insignificant differences were observed between the chromatographic behavior of cysteyl and selenocysteyl tRNA on RPC-5 columns. Either *E. coli* lacks this species of tRNA or the chromatographic shifts reported by these authors were significant after all.

Because of the low recoveries of ^{75}Se-labeled selenocysteine derivatives from tRNA, it is possible that as much as 61% of the ^{75}Se in tRNA

prepared from [^{75}Se]selenocysteine is liver slices is in a form other than selenocysteine. However, considereing the complete lability of the ^{75}Se in tRNA to ribonuclease, alkaline pH and NH_2OH, it seems likely that most of the ^{75}Se is present as the amino acid aminoacylated to tRNA.

Although the existence of a specific selenocysteyl tRNA is consistent with the idea that selenocysteine is incorporated intact into selenoproteins during protein synthesis, is does not prove this point. What these data do show is that selenocysteyl tRNA possesses the qualities required of an intermediate in such a pathway. It remains to be determined if this type of pathway occurs in rat liver in vivo and, if it does, to what extent it could account for the synthesis of known selenocysteine-containing proteins such as glutathione peroxidase [11]. This laboratory is conducting further research into the important question of whether selenocysteine is incorporated into proteins at a specific site during protein synthesis or by a post-translational mechanism.

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